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Authors

Kaysen, GA
Chou, LY
Humphreys, MH

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REQUIREMENT OF Zn TO DEMONSTRATE HCO₃-STIMULATED ATPase ACTIVITY OF RAT SMALL INTESTINAL BRUSH BORDER

G. A. KAYSEN, L. Y. CHOU, and M. H. HUMPHREYS

From the University of California Renal Center and Department of Medicine, San Francisco General Hospital, San Francisco, California 94110

ABSTRACT

The existence of a membrane-bound HCO₃-stimulated ATPase in intestinal mucosa is controversial. A crude brush border fraction of rat small intestinal homogenates contained HCO₃-ATPase activity which was inhibited by preincubation with 3 mM EDTA. Alkaline phosphatase activity of this preparation was also inhibited in a parallel, time-dependent fashion by preincubation with EDTA. When 5 mM ZnSO₄ accompanied 3 mM EDTA in the preincubation mix, preservation of both enzyme activities occurred, demonstrating a requirement of Zn for the activity of both these phosphatases.

These studies support the earlier contention that HCO₃-ATPase and alkaline phosphatase activities may be different properties of the same enzyme, and raise the possibility that the ATPase could play a role in intestinal ion transport. The failure to identify a membrane-bound HCO₃-ATPase by other workers could be due to the exposure to EDTA which occurred in their tissue preparation.

KEY WORDS enzyme activation · anions · ion transport · enzyme inhibition

Controversy exists concerning the subcellular localization of anion-stimulated ATPase activity in epithelial tissues such as kidney, intestine, and stomach. In the case of small intestine, van Os et al. (11) found that virtually all HCO₃-ATPase activity co-purified with the mitochondrial marker enzyme succinic dehydrogenase, leading them to conclude that little if any activity was associated with the brush border in rat intestine. However, recent results from this laboratory identified HCO₃-stimulated ATPase activity in a brush border fraction of rat small intestine (6), and strongly suggested that this activity was a property of intestinal alkaline phosphatase in both rat and cow (6, 7). Since alkaline phosphatase is primarily, if not exclusively, associated with the brush border

(9, 10), a mitochondrial origin of this HCO₃-ATPase seems very remote, particularly since little alkaline phosphatase activity is associated with the mitochondrial fraction. Our results lend credence to the argument that at least one species of HCO₃-ATPase is associated with the external cell membrane, most likely the brush border, and would therefore support the contention that this enzyme may have a direct role in transport across the rat small intestine. Our findings thus are in apparent contradiction with those of van Os et al. (11). Since EDTA was used in the subcellular fractionations performed by van Os et al., as well as in the assay for HCO₃-ATPase, we have studied the inhibitory effect of EDTA on intestinal brush border alkaline phosphatase and HCO₃-ATPase activities in the presence of excess Mg to explain the apparent discrepancy between their data (11) and our own (6).

MATERIALS AND METHODS

Tissue Preparation

Crude brush borders from rat jejunum were prepared as previously described (6). This resulted in a 15-fold enrichment of alkaline phosphatase and sucrase activities over activities in whole tissue homogenates without appreciable contamination by the mitochondrial marker enzyme succinic dehydrogenase. $\text{HCO}_3\text{-ATPase}$ in this brush border preparation was enriched in parallel with alkaline phosphatase (6).

Enzyme Assays

$\text{HCO}_3\text{-ATPase}$ activity was measured according to the method of van Os et al. (11). The assay tubes contained, in mmol/l, Tris-HCl buffer (pH 8.5), 50; MgCl_2 , 5; EDTA, 3; Na_2ATP , 3; and NaHCO_3 or NaSCN , 100, in a total vol of 1 ml. The reaction was started by adding 25–50 μg of purified brush border (PBB) protein. Tubes were incubated in a water bath at 37°C for 15 min, after which the reaction was stopped by addition of 0.2 ml of 30% trichloroacetic acid. The reaction mixture was then centrifuged at 6,000 g for 5 min, and 1.0 ml of the deproteinized supernate was analyzed for inorganic phosphorus by the method of Fiske and SubbaRow (5). Since the slope of the line relating optical density to Pi concentration differed in SCN- and $\text{HCO}_3\text{-}$ containing solutions, standards were made in 100 mM solutions of these ions and the optical density of the assay tubes was related to Pi concentration using the appropriate standard curve. $\text{HCO}_3\text{-ATPase}$ was calculated as the difference between the rate of ATP hydrolysis in the presence of $\text{HCO}_3\text{-}$ and the rate in the presence of SCN (11). Results are expressed as $\mu\text{mol Pi/mg protein/h}$; protein was measured according to the method of Lowry et al. (8) using bovine serum albumin as standard. Alkaline phosphatase was measured according to the method of Bowers and McComb (2).

Effect of EDTA

To study the effect of EDTA on these enzyme activities, PBB protein was preincubated for various lengths of time with EDTA; all preincubations were carried out at 0°C . For alkaline phosphatase activity, both 3 mM and 30 mM EDTA in the preincubation mix were tested while in the case of $\text{HCO}_3\text{-ATPase}$ activity, only preincubation with 3 mM EDTA was used. These experiments were repeated with 5 mM ZnSO_4 accompanying preincubation with 3 mM EDTA to study any protective effect of added Zn. In these experiments, assay conditions were as otherwise described above; in particular, MgCl_2 was present when effects of ZnSO_4 were studied.

RESULTS

Preincubation of PBB protein with EDTA caused a progressive reduction in alkaline phosphatase activity with time. This was more pronounced with

30 mM EDTA than with 3 mM; with 90-min preincubation, 90% of basal activity (7.21 ± 0.16 [SE] $\mu\text{mol/mg/min}$) was inhibited, while 60% inhibition was achieved with 3 mM EDTA. When preincubation of PBB with 3 mM EDTA was carried out in the presence of 5 mM ZnSO_4 , substantial protection of enzyme activity resulted; activity after 90-min preincubation was 92% of basal activity. These findings are consistent with previous demonstrations that Zn is an essential constituent for the activity of alkaline phosphatase.

The effects of preincubation of PBB with 3 mM EDTA on $\text{HCO}_3\text{-ATPase}$ activity are shown in Fig. 1. Aliquots of the enzyme were taken out of the preincubation mixture at the times indicated in the figure and assayed for $\text{HCO}_3\text{-ATPase}$ as described above. Considerable $\text{HCO}_3\text{-ATPase}$ activity was present with very short preincubation times, but a decrease in activity of the enzyme was noted as preincubation time increased. The presence of excess Mg in the incubation mixture did not protect against this inactivation.

The protective effect of preincubation with Zn on $\text{HCO}_3\text{-ATPase}$ activity is demonstrated in Table I. With no preincubation with either Zn or EDTA, considerable enzyme activity was present (condition A), even with 3 mM EDTA present in the incubation mix. Preincubation of PBB with 3 mM EDTA for 95 min reduced $\text{HCO}_3\text{-ATPase}$ activity to one-third the control value (condition

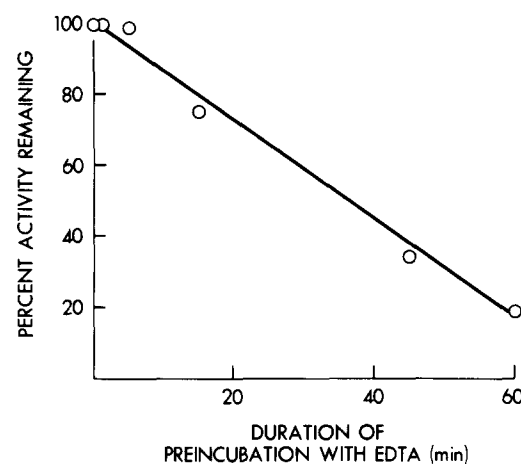


FIGURE 1 Inhibition of $\text{HCO}_3\text{-}$ stimulated ATPase activity of intestinal brush border by preincubation with 3 mM EDTA. Aliquots of preincubation mix were removed at the indicated times and assayed for $\text{HCO}_3\text{-ATPase}$ activity as described in Materials and Methods. Specific activity before preincubation was $111.5 \mu\text{mol Pi} \cdot \text{mg}^{-1} \cdot \text{hr}^{-1}$.

TABLE I
Effect of Preincubation with EDTA and ZnSO₄ on
HCO₃-ATPase Activity of PBB Protein*

Condition	Preincubation with 3 mM EDTA (min)	ZnSO ₄ in preincuba- tion mix (mM)	Specific activity ($\mu\text{mol Pi} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$)
A	0	0	182.5 \pm 10.0
B	95	0	65.7 \pm 03.8 [‡]
C	0	5	195.4 \pm 14.0
D	95	5	221.6 \pm 15.0 [§]

* Values are means \pm 1 SD of three measurements for each condition. HCO₃-ATPase activity was determined as described in Materials and Methods. Preincubation was carried out at 0°C.

[‡] Significantly less than A, C, or D ($p < 0.001$ by unpaired t test).

[§] Significantly greater than A ($p < 0.02$ by unpaired t test).

B), indicating that lengthy exposure to EDTA is required for inhibition to take place. Assay for enzyme activity in the presence of 5 mM ZnSO₄ but without prior incubation in EDTA did not appreciably alter enzyme activity (condition C), while preincubation in 3 mM EDTA in the presence of 5 mM ZnSO₄ resulted in actual enhancement of activity (condition D). Thus, Zn⁺⁺ exerted a protective effect against the inhibition of HCO₃-ATPase caused by EDTA, just as it did for alkaline phosphatase.

DISCUSSION

The results of the present study clearly demonstrate that EDTA inhibits both alkaline phosphatase and HCO₃-ATPase activities of an intestinal brush border preparation in a time-dependent manner. Furthermore, they show that incubation in the presence of excess Zn ion markedly protects enzyme activity from inhibition by EDTA. These findings therefore extend the earlier observations which suggested that alkaline phosphatase and HCO₃-ATPase activities could be different properties of the same enzyme (6, 7). Alkaline phosphatase is a Zn-containing metalloprotein which requires Zn for maintenance of enzyme activity (3). EDTA is a known inhibitor of alkaline phos-

phatase, probably acting by chelation of Zn at the active site (1, 4). The present results demonstrate that this inhibition of EDTA is largely prevented if excess Zn accompanies the exposure to EDTA. HCO₃-ATPase activity has been associated with alkaline phosphatase (6, 7); the finding that EDTA inhibits both HCO₃-ATPase and alkaline phosphatase activities, and that this inhibition can be prevented by Zn in both cases, further supports this contention. Zn consequently appears necessary for either manifestation of this phosphatase enzyme.

It seems probable that the failure of van Os and colleagues to demonstrate HCO₃-ATPase activity in their brush border fraction is due to the exposure to EDTA which occurred in the process of tissue preparation. In this respect, it is noteworthy that Zn was added back to tissues for measurement of alkaline phosphatase activity, but not for HCO₃-ATPase activity in their studies (11).

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